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Systemically available bone morphogenetic protein2 and 7 effect bone metabolism

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ABSTRACT

Purpose: Bone morphogenetic protein (BMP)2 and 7 are used in patients with long bone fractures and non-unions, and spinal fusions. It is unknown whether their potential systemic bioavailability following an local bone administration might effect the skeleton metabolism. To answer this question we examined effects of systemically administered BMP2 and 7 on bone in a newly developed rat model with low level of calciotropic hormones.

Methods: Removal of thyroid and parathyroid glands (TPTx) in rats resulted in decreased level of calciotropic hormones and a subsequent bone loss assessed by microCT and measurement of serum bone formation and resorption markers, including osteocalcin, C-telopeptide, osteoprotegerin and RANKL. Results were complemented with *in vitro* studies on osteoblast and osteoclast activity by both BMP2 and 7. The doses used have been calculated from published pharmacodynamic (PD) and bioavailability results from pre-clinical BMP2 and 7 studies.

Results: TPTx resulted in bone loss which was restored by systemic administration of 10-70 µg/kg of BMP2 and 10-250 µg/kg of BMP7. BMP2 showed a higher capacity for enhancing trabecular microarchitecture, while BMP7 augmented trabecular thickness. *In vitro* experiments revealed that BMP2 and 7, when uncoupled, increased the number and activity of both osteoblasts and osteoclasts.

Conclusions: Surprisingly, both BMP2 and 7 showed an increased bone volume in an *in vivo* environment of low calciotropic hormones. Locally administered BMP2 and 7 from bone devices might become partially available in circulation but will not mediate a systemic bone loss.

Key words: bone morphogenetic protein 2, bone morphogenetic protein 7, microCT, bone volume

INTRODUCTION

Bone morphogenetic proteins (BMPs) play an important role in the bone and joint regeneration [1-4]. BMP2 and 7 with the bovine collagen carrier were approved for the treatment of spinal fusions, long bone non-unions and acute fractures after randomized, controlled and blinded clinical trials [5,6]. The osteoinductive ability of BMP 2 and 7 distinguishes them from other available bone grafts and eliminates the donor-site morbidity in contrast to autologous bone grafting. Moreover, no adverse events were observed in more than 700 patients from randomized clinical trials (RCTs). Recently, both Infuse and BMP7 based Osigraft were confronted with side-effects and unresolved clinical [7,8]. Specifically, vertebral implant migration and subsidence as a result of early osteolysis, heterotopic ossification and retrograde ejaculation were among registered complications [7,8]. Similar, but more pronounced side effects occurred when BMP 2 and 7 were used for the treatment of long bone fractures and non-unions, especially at sites where the bone was exposed directly under the skin, like in distal radius fracture patients [9]. These results were further supported by recent preclinical findings of endosteal site resorption after application of BMP7 around femoral prostheses in large animals [10]. To avoid similar unwanted misapprehensions, an appropriate guideline of drug discovery aimed for accelerating bone healing was proposed for the non-clinical development [11].

In formal regulatory non-clinical PD, toxicity and safety evaluation BMP2 and 7 showed practically identical properties: bone healing effect when applied orthotopically with low ($\leq$ 2-3%) absolute systemic bioavailability and virtually no systemic toxicity/effects in safety pharmacology studies with systemic administration. However, it is estimated that BMPs applied locally remain active for at least 2 weeks suggesting that some amount might remain systemically available for the same period of time. We used a range of BMP2 and 7 concentrations to mimic their systemic availability following local application in patients, using a newly developed rat model with low level of calciotropic hormones.

MATERIALS AND METHODS

Animal model and study protocol

Four and six months old Sprague-Dawley rats, weighting 350-380g, were subjected to TPTx. Rats were anesthetized with an intraperitoneal injection of rompun (Xylapan 0.6 ml/kg) and ketamine (Narketan 0.8 ml/kg). Surgery was performed by ventral approach - skin and pretracheal muscles were gently dissected, following by removal of thyroid and parathyroid glands using a surgical blade No 15 [12]. Hemostasis was achieved and wound was subsequently closed in anatomical layers. Six animals per experiment were subjected to sham surgery during which upper muscle layers were dissected, but thyroid and parathyroid glands remained intact. After the surgery, the rats were kept on a regular chow and 1% calcium gluconate water. Animals were divided into eight groups (n=6): (1) sham; (2) TPTx; (3) TPTx + BMP2 10 μg/kg/day, (4) TPTx + BMP2 70 μg/kg/day, (5) TPTx + BMP2 250 μg/kg/day, (6) TPTx + BMP7 10 μg/kg/day, (7) TPTx + BMP7 70 μg/kg/day, (8) TPTx + BMP7 250 μg/kg/day. Rats were kept in standard conditions for seven days before treatment. Therapy was administered intraperitoneally for 14 days, starting from seventh day after surgery (Fig 1A). All experiments and protocols were approved by the Institutional Animal Care Committee of Medical Faculty, University of Zagreb and Ministry of Science and Technology.

Recombinant human BMPs

Commercially available recombinant human BMP2 and BMP7 (R&D Systems, Minneapolis, MN) were administered intraperitoneally from 7^{th} to 21^{th} day of the experiment according to the protocol at the three different doses.

Biochemical Serum Parameters

Blood (0.5 ml) was collected at various time points by tail vein puncture for serum chemistry (calcium and phosphorus measurements) and for determination of bone turnover biomarkers (osteocalcin and C-telopeptide). Animals were lightly anesthetized during the bleed procedure with CO_2/O_2. Chemistry endpoints were analyzed using a Hitachi 917 auto-analyzer (Roche, Indianapolis, IN). Serum bone formation and resorption markers were measured by commercially available kits. Serum concentration of osteocalcin was measured by ELISA using rat osteocalcin EIA kits (Biomedical Technologies Inc., Stoughton, MA). Serum concentration of C-telopeptide was measured by ELISA using RatLaps ELISA kits (Nordic Bioscience Diagnostics, Herlev, Denmark). The osteoprotegerin (OPG) level in

serum was measured by ELISA using the Biomedica rat OPG ELISA kit (Biomedica, Wien, Austria). The receptor activator of nuclear factor kappa-B ligand (RANKL) level in serum was measured by ELISA using the Biomedica rat RANKL ELISA kit (Biomedica, Wien, Austria). The minimum detectable concentration of osteocalcin, CTx, RANKL and OPG was 1 ng/ml, 2 ng/ml, 0.016 pmol/liter and 0.73 pmol/liter respectively.

MicroCT

The μCT 1076 and analysing software used in these experiments were obtained from SkyScan (Kontich, Belgium). The distal femur was scanned in 250 slices, each 18 μm thick in the dorsoventral direction. 3D reconstruction of the bone was performed using the triangulation algorithm (NRecon). The trabecular bone parameters including trabecular bone volume (BV, mm^3), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, μm) and trabecular spacing (Tb.Sp, μm) were directly measured on 3D images.

Cell culture

Bone marrow cells were harvested from femurs and tibias of 3 months old rats previously subjected to TPTx, pooled and plated on 24-well plates at density of $1x10^6$ cells per well. Differentiation media for osteoclasts contained α-MEM, 10% FBS, macrophage colony-stimulating factor (M-CSF, 20 ng/mL; Sigma Aldrich), and recombinant human soluble receptor activator of nuclear factor-κB ligand (RANKL, 20 ng/mL; Sigma Aldrich). BMP2 (100 ng/mL) and BMP7 (100 ng/mL) were added to the medium on day 1 and replaced at every feeding until termination on day 6. The cells were fixed with 4% paraformaldehyde, and adherent osteoclasts were identified by tartrate-resistant acid phosphatase (TRAP) staining using a commercially available kit (Sigma Aldrich). Only osteoclasts with three or more nuclei were counted. For RNA isolation and qRT-PCR analysis cells were collected in TRI Reagent (Life Technologies).

Differentiation medium for osteoblasts was added on day 7 and contained α-MEM, 10% FBS, 10mM β-glycerophosphate and 50 μg/mL ascorbic acid.. The medium was changed every two days until the culture was terminated on day 19. BMP2 (100 ng/mL) or BMP7 (100 ng/mL) were added to the medium at every feeding. Osteoblasts were identified by alkaline phosphatase stain using a commercially available kit (Sigma Aldrich). Von Kossa staining was used to determine the mineralized matrix formation. Quantification of the mineralized area was performed using S-form software and expressed as percentage of the mineralized area.

qPCR

Total RNA from osteoclasts was isolated using TRIreagent (Ambion) according to manufacturer's instructions. 1 μg of total RNA was used in reverse transcription which was perfomed using High-capacity cDNA reverse transcription kit (Applied Biosystems) and oligo-dT primers for mRNA transcription. For gene expression analysis, quantitative real-time PCR was performed on a Light Cycler (Roche Applied Science) using SYBR Premix Ex Taq^{TM} II (TaKaRa). The comparative C_t method ($\Delta\Delta C_t$) was used for relative quantification of gene expression [13], with β-actin as a reference gene. Sequences of primers used in PCR were as follows: β-actin FOR: 5'-GCGCAAGTACTCTGTGTGGA-3', REV: 5'-ACATCTGCTGGAAGGTGGAC-3'; TRAP FOR: 5'-ACGCCAATGACAAGAGGTTC-3', REV: 5'-AGGTGATCATGGTTTCCAGC-3'; cathepsin K FOR: 5'-AGACGCTTACCCGTATGTGG-3', REV: 5'- CACTGCTCTCTTCAGGGCTT-3'. All primers were designed using Primer3 software (http://simgene.com/Primer3). Results are represented as a fold change of the comparative expression level with standard deviation.

Statistical analyses

Data from biochemical, biomechanical and μCT analysis as well as *in vitro* tests are presented as mean ± standard deviation (SD). P-value is for simultaneous comparison between groups in respect to all indicators (multivariate analysis of variance). All individual indicator P-values were either <0.001 or <0.05 as indicated in figures.

RESULTS

BMP 2 and 7 enhanced bone remodeling in TPTx rats

Following surgical removal of thyroid and parathyroid glands (TPTx) animals experienced hypocalcaemia and hyperphosphataemia, as previously described [Dumic-Cule I et al 2014]. PTH, T3/T4, calcitonin and $1,25(OH)_2D_3$ plasma levels at 7 day recovery period were very low. Rats were then treated for 14 days with 3 doses of BMP2 and 7. This model enabled evaluation of BMP2 and 7 direct bone effects in absence of calciotropic hormones (Fig 1). T3/T4 and PTH plasma concentration were significantly decreased, while thyrotropin (TSH) was increased due to the negative T3/T4 feedback loop. $1,25(OH)_2D_3$ level was low as a consequence of PTH deficiency. These changes resulted in low serum calcium level when compared to sham animals, associated with reduced bone resorption (Fig 2A). BMP2 showed a significant linear and quadratic trend in increasing calcium serum level, while BMP7 had a significant linear trend (Fig 2A). The overall calcium augmentation was significantly higher for BMP2 (Fig 2A). As expected, the phosphate level was in a good correlation with calcium, maintaining the calcium-phosphate product with a similar trend between BMP2 and 7 doses (Fig 2B). Osteocalcin and C-telopeptide, serum markers of bone formation and resorption, were both suppressed in TPTx animals, confirming a low remodeling rate (Fig 2C, D). The osteocalcin plasma level in TPTx rats was significantly lower than in sham animals (Fig 2C). Neither BMP2 nor BMP7 therapy had a significant osteocalcin trend (Fig 2C). However, there was an overall significant difference between BMP2 and 7 across all doses in favor of BMP7 (Fig 2C). On the contrary, plasma C-telopeptide level was lower in TPTx rats with both a significant linear and quadratic trend for all BMP2 doses. BMP7 had a significant linear trend, while the quadratic trend was insignificant. There was no overall difference between BMP2 and 7 therapy (Fig 2D).

BMP 2 and 7 partially restored the bone loss following TPTx

A significant bone loss occurred within 7 days after TPTx: bone volume/tissue volume (BV/TV) decreased in TPTx rats (9,66 ± 2,24) as compared to sham animals (12,02 ± 1,77) (Fig 3). Following 14 days of BMP2 and 7 administration μCT of the distal femur was performed to determine their capacity to compensate for TPTx caused bone loss. There was a significant linear and quadratic trend across BMP2 doses, while BMP7 had only a significant linear trend (Fig 3B). Individually, the highest dose of BMP2 (250 μg/kg) enhanced BV/TV by 15%, reaching the bone volume similar to TPTx animals (Fig 3B). The Tb.N. was

increased by 37% and 58%, respectively in groups treated with 10 µg/kg and 70 µg/kg of BMP2, and only a moderate increase was observed when the highest BMP2 dose was administered. The Tb.Th. remained lower in rats treated with BMP2 (Fig 3C). In TPTx animals treated with 10 µg/kg, 70 µg/kg and 250 µg/kg of BMP7, the BV/TV was increased by 14%, 38% and 52%, respectively, as compared to TPTx control animals (Fig 3B). The Tb.N. and Tb.Th. were increased in BMP7 treated animals irrespective of the dose.

Both BMP2 and BMP7 lowered soluble RANKL

Plasma samples from *in vivo* studies have been analyzed for circulating soluble RANK ligand (sRANKL) and osteoprotegerin (OPG) at day 14 of therapy. Treatment with both BMP2 and BMP7 had no trend in plasma levels of sRANKL (Fig 4) and OPG (data not shown).

***In vitro* effects of BMP2 and BMP7**

After 6 days of treatment both BMP2 (100 ng/mL) and BMP7 (100 ng/mL) increased the number of primary rat osteoclasts isolated from TPTx rats on day 7 (Fig 5). Surprisingly, BMP2 and 7 did not stimulate catepsin K expression (data not shown). Consistent with *in vivo* results, both BMP2 and 7 enhanced the differentiation of bone marrow derived mesenchymal stem cells towards an osteoblastic phenotype as evidenced by increased bone mineral nodule formation as compared to control animals (Fig 5). This suggested that BMP2 and 7 significantly promote both bone formation and resorption in cultured TPTx bone cells. However, their effective outcome in different clinical indications will depend on factors in the specific bone environment, where osteoblasts are coupled to osteoclasts [14]. Therefore, periosteal and muscle satellite progenitors are the major BMP target for uncoupled bone formation activity, mimicking the situation as observed in this study.

DISCUSSION

The potential of systemically administered BMP2 and 7 to enhance the bone volume was assessed in the new TPTx rat osteoporotic model. BMP2 was more beneficial at lower doses, while the effect of BMP7 was increased at higher doses. Moreover, both BMP2 and 7 did not restore the bone volume to the pre-operative values. Thyroid hormone relation with the BMP system is unknown, while the role of T3/T4 in bone metabolism has been extensively investigated. The role of BMPs in thyrotropin signaling has been suggested namely, BMP2, 4, 6 and 7 suppressed the TSH receptor mRNA expression in thyrocytes [15].

Osteolysis and heterotopic ossification as major side-effects of BMP2 and 7 devices at local sites of implantation have not been fully elucidated. Currently available BMP devices contain milligram amounts of BMP2 and 7 which form protein aggregates at the neutral pH. Those agglomerates usually disseminate in the adjacent tissue and subsequently potentiate heterotopic ossification via stimulating uncoupled mesenchymal osteoprogenitor cells outside the bone cavity. Endosteal site osteolysis was initially noticed when BMP devices were used on sites under the skin with frail muscle envelope. Ekrol et al unanimously showed superiority of autogenous bone graft over BMP devices when used in both external and internal fixation of distal radius corrective osteotomy [9]. This was additionally supplemented with preclinical finding of an increased trabecular bone mass in mice without BMPIa receptor, which was explained with receptor activator of nuclear factor kappa-B (RANK) - receptor activator of nuclear factor kappa-B ligand (RANKL) – osteoprotegerin (OPG) pathway suppressed osteoclastogenesis [16]. Osteoblastic effects on osteoclastogenesis were explained by discovering RANK-RANKL-OPG system, while vice-versa effect of osteoclasts on osteoblasts remained unclear [17]. It has been recently reported that platelet derived growth factor-BB (PDGF-BB) is a promising candidate for osteoclast - osteoblast interplay, which could complete the coupling circle of bone remodelling [18]. Therefore, the coupling mechanism of osteoblast-osteoclast interaction is probably responsible for orthotopic site osteolysis in BMP treatment which overbalanced its osteoinductive ability. BMP2 enhanced the trabecular bone quality, indicating that a low dose was sufficient for this effect. Therefore, large amount of BMP2 in commercial devices might increase the unwanted side effects.

The expected bioavailability of BMP2 and 7 used in commercially available bone devices is low; below the level of 5% of the administered dose, which is eventually close to the highest dose of BMP2 and the middle dose of BMP7 used in these experiments. Thus, these results surprisingly suggest that small amounts of BMP2 and 7 eventually entering the

circulation following therapy of bone injuries will not stimulate generalized bone loss. In support of these findings we have previously demonstrated that intravenously administered rhBMP6 restored lost bone by stimulating bone formation in osteoporotic ovariectomized aged rats [19].

In conclusion, systemic administration of BMP2 and 7 during 14 days in TPTx rats proved their osteoinductive potential and did not confirm the osteolytic effect observed in human BMP devices suggesting that when BMP2 and 7 are used on orthotopic site their effectiveness will depend on the bone cells microenvironment and the preservation of surrounding tissues, mainly periosteum and skeletal muscle satellite cells. Since there is a great medical need for a more effective and safe treatment option in bone defect regeneration we have lately introduced a novel bone healing principle based on using low levels of BMP6 and a carrier prepared from patient's own blood [Vukicevic et al 2014].

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FIGURE DESCRIPTION

Figure 1: Outline of animal experiments. Animals were submitted to total thyreoidectomy-parathyreoidectomy (TPTx) or sham surgery. After a 7-day recovery period, the effect of surgery was verified by reduced serum levels of parathormone (PTH), thyroxine (T4) and calcitriol ($1,25(OH)_2D_3$). Subsequently, TPTx animals were submitted to a 14-day treatment with BMP2 or BMP7 (10, 70 or 250 μg/kg/day).

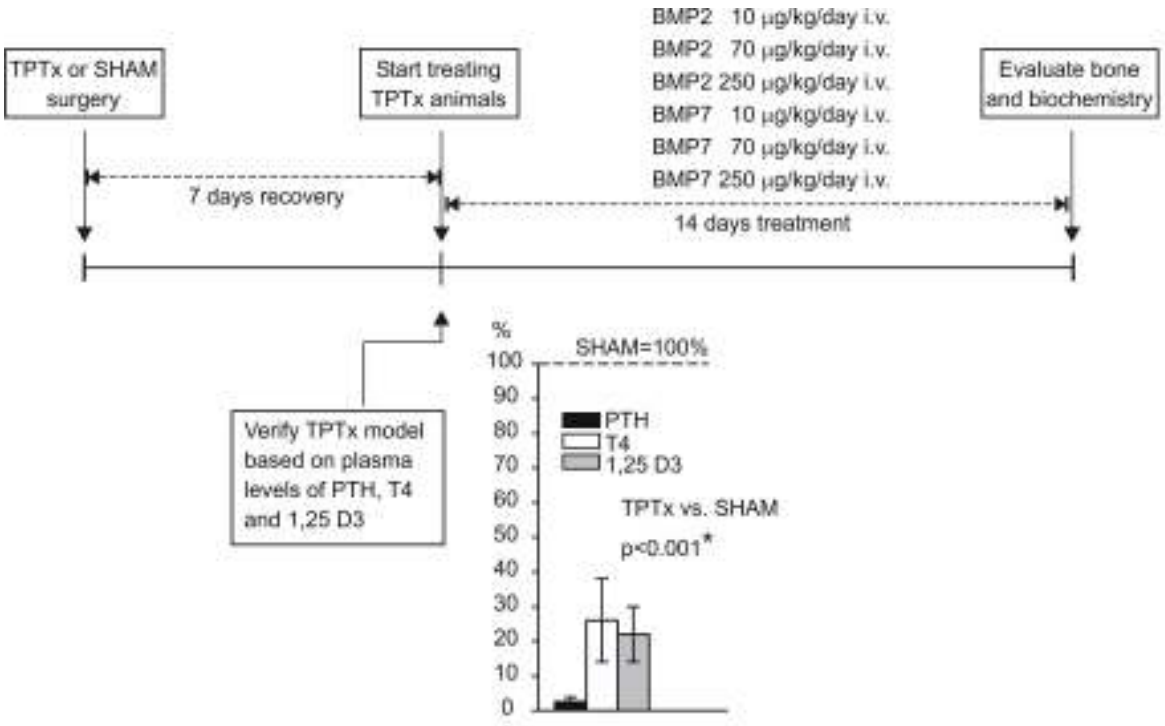


Figure 2: Serum level of calcium (A), phosphate (B), osteocalcin (C) and C-telopeptide (D) in sham and thyroparathyroidectomized rats before and after treatment with different amounts of BMP2 and 7.

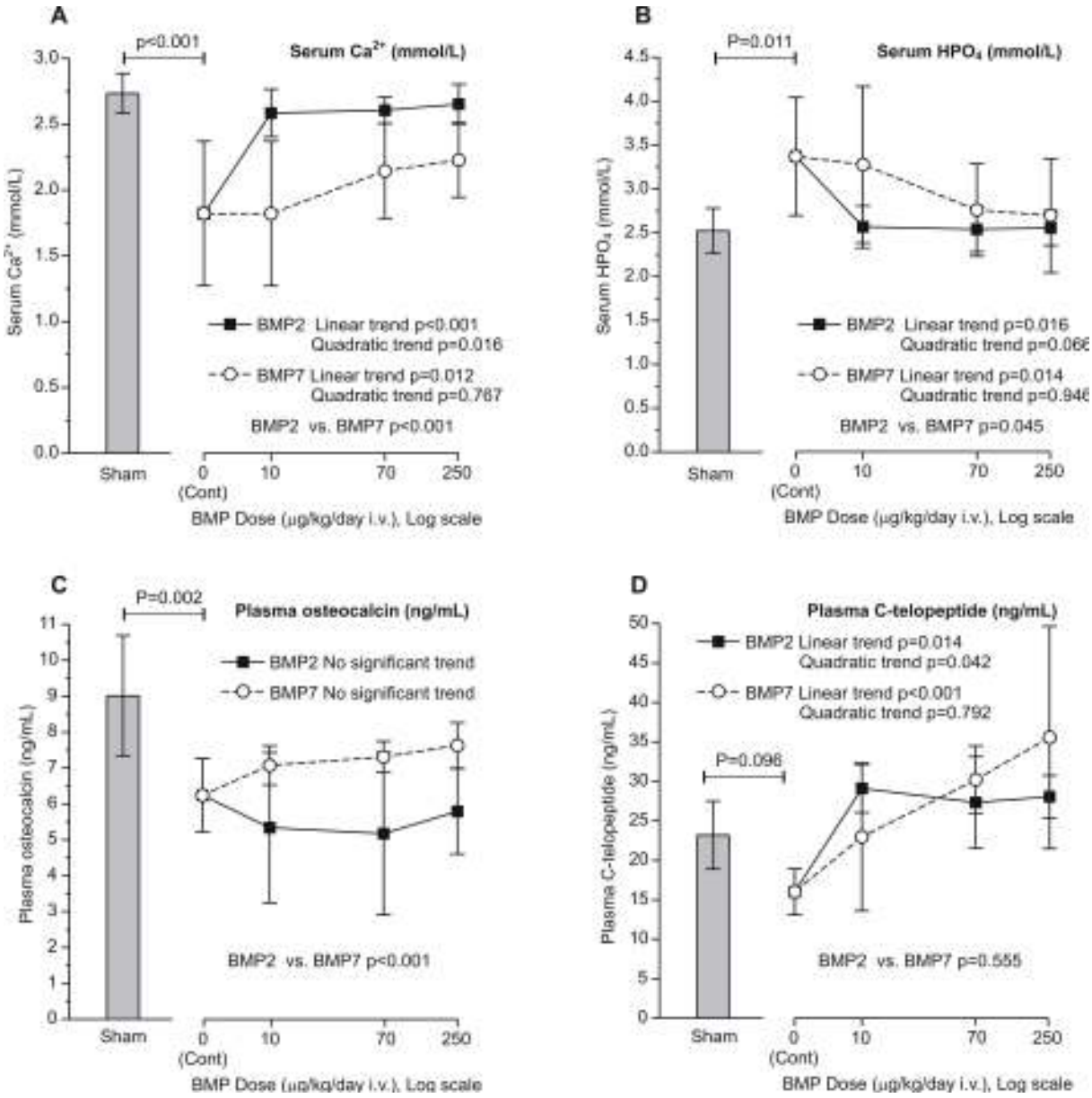
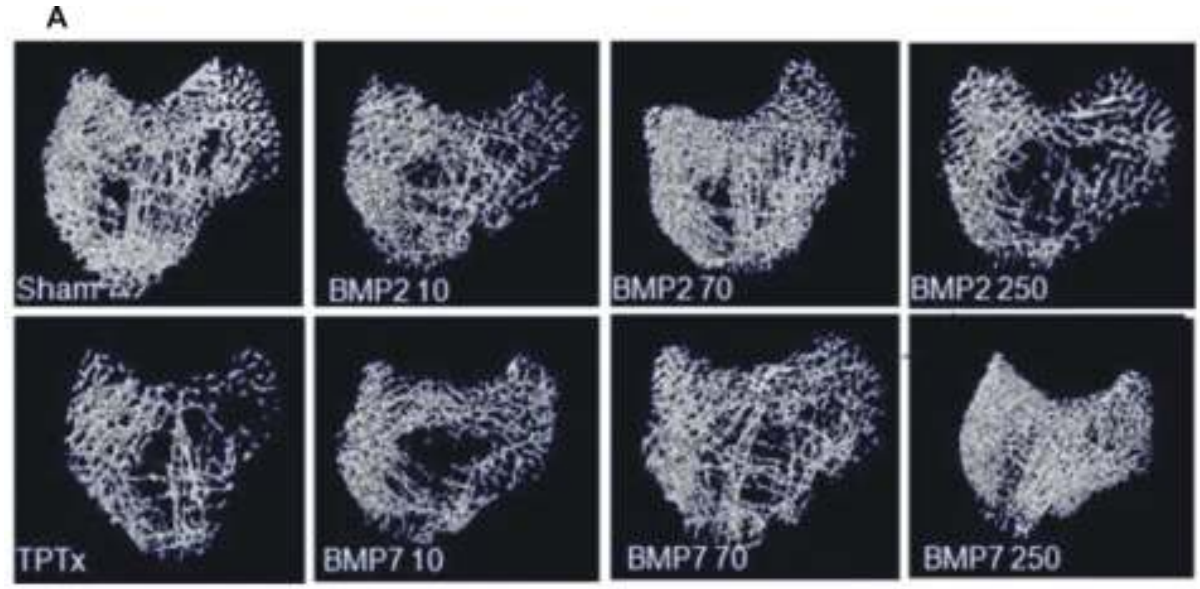
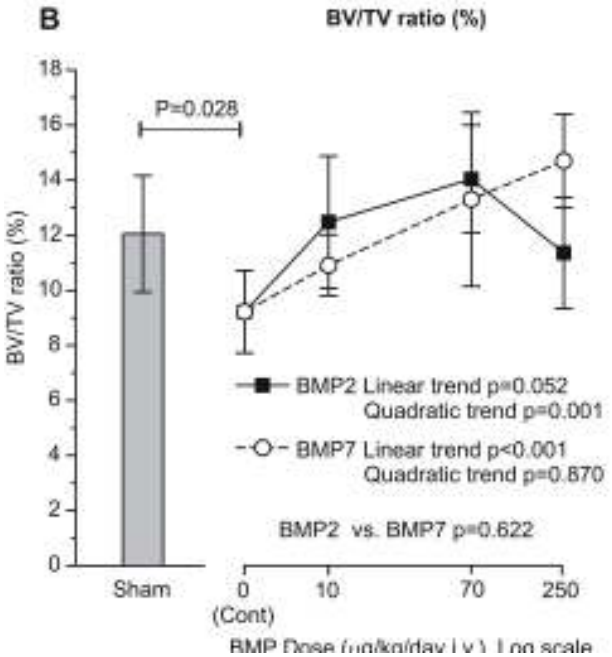


Figure 3: Bone volume/tissue volume measured by microCT in sham and thyroparathyroidectomized rats before and after treatment with different concentrations of BMP2 and 7 (B). MicroCT image of distal femur of a representative rat from each group is shown (A). Systematic outline of trabecular bone parameters of distal femurs: Tb.N. (1/mm), Tb.Th. (μm) (C).





C

Group (μg/kg/day)	Tb.N.	Tb.Th.
Sham	0,00240 ± 0,00188	60,65 ± 15,41
TPTx	0,00134 ± 0,00034	72,66 ± 7,85
BMP2 10	0,00184 ± 0,00035[a]	62,21 ± 1,97[a]
BMP2 70	0,00212 ± 0,00024[a]	60,29 ± 2,18[a]
BMP2 250	0,00165 ± 0,00037	61,62 ± 2,80[a]
BMP7 10	0,00158 ± 0,00021	82,37 ± 10,04[b]
BMP7 70	0,00176 ± 0,00039	87,94 ± 5,43[ab]
BMP7 250	0,00200 ± 0,00029[a]	84,52 ± 7,22[ab]

Figure 4: Serum level of soluble RANKL in sham and thyroparathyroidectomized rats before and after treatment with different amounts of BMP2 and 7.

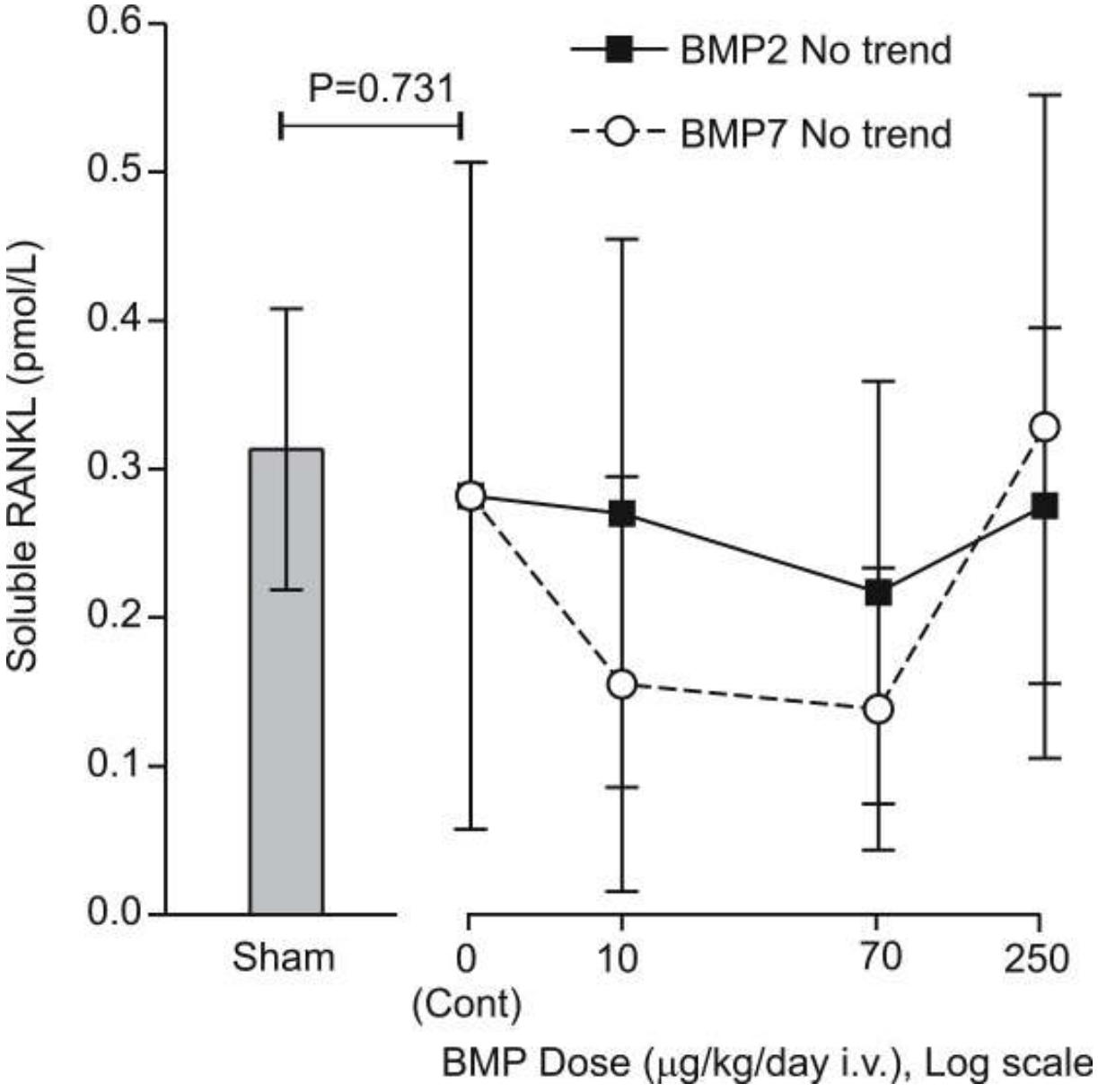


Figure 5: Effects of BMP2 and 7 on osteoclast and osteoblast differentiation. The number of TRAP+ osteoclasts was determined. Significant difference is indicated with respect to cultivated cells without treatment (A). Alkaline phosphatase staining was used to assess effect of BMP2 and 7 on osteoblast differentiation. Area of alkaline phosphatase was calculated by histomorphometry (B).